ARL-TR-7645 ● APR 2016

US Army Research Laboratory

Comparison of 2 Luminex-based Multiplexed Protein Assays for Quantifying Microglia Activation and Inflammatory Proteins

by Lee Campbell, Emily Wires, Brandon Harvey, and Keith Whitaker

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED (From - To)
April 2016	Final	November 2015–January 2016

4. TITLE AND SUBTITLE
Comparison of 2 Luminex-based Multiplexed Protein Assays for Quantifying Microglia Activation and Inflammatory Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Lee Campbell, Emily Wires, Brandon Harvey, and Keith Whitaker

5d. PROJECT NUMBER
DR16-HR-023

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
US Army Research Laboratory
ATTN: RDRL-HRT-M
Aberdeen Proving Ground, MD 21005-5425

8. PERFORMING ORGANIZATION REPORT NUMBER
ARL-TR-7645

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The complex injury cascades associated with mild traumatic-brain injury partly involve inflammation in the brain that is triggered by the release of chemokines and cytokines from the brain's resident immune cells, microglia. The technical ability to quantify these small molecules from biological samples is critical toward new technology that limits the damage following a concussive event. This project's objective was to test the feasibility of using multiplexed protein assays based on Luminex microspheres to fulfil the requirement to quantify inflammatory proteins from biological samples derived from rats. Two vendors, R&D Systems and EMD Millipore, market antibodies conjugated with Luminex fluorescently labeled microspheres that can be used to quantify inflammatory biomarkers directly from samples of plasma, cell cultures, tissue homogenates, cerebral spinal fluid, and other tissues. The single kit from R&D Systems removed many sources of human error and provides a diluent to generate a single standard curve for a variety of sample types. EMD Millipore has many more products available to detect a wide range of analytes in rat samples, but these products are separated into different kits. Without optimization, the products from both vendors were only capable of relative quantification of fluorescence instead of true quantification of concentration.

15. SUBJECT TERMS
multiplexed protein assay, TBI, traumatic brain injury, luminex, blunt-trauma brain injury

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Keith W Whitaker
Unclassified	Unclassified	Unclassified	UU	30	19b. TELEPHONE NUMBER (Include area code) 410-278-5599

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1. Introduction

Traumatic brain injuries (TBIs) are a problem for the US Army (Logan et al. 2013; Hoge et al. 2008) due to combat and work-related concussions as well as Soldiers' risky behaviors post-deployment (Kelley et al 2012). Coupled with the growing interest in sports-related TBIs, there is a demand for medicine and biotechnology to develop methods capable of limiting the negative effects of concussive injuries. The progression of a blunt-trauma brain injury has been well described elsewhere (Blennow et al. 2012). Briefly, there are primary effects from the physics of the injury that cause damage and secondary effects as the body's response to the primary injury generates inflammation and edema. The US Army Research Laboratory (ARL) is invested in developing technology to limit the primary effect of concussion by reducing the initial physical forces that cause trauma (Vargas-Gonzalez and Gurganus 2015). Limitation of the secondary effects has traditionally been the focus of medical research, but a recent project in ARL aims to develop biotechnological tools capable of altering cellular function of the brain's immune cells (e.g., microglia) by decreasing their production of proteins that lead to inflammation. If successful, this has the potential to reduce 2 leading causes of secondary injury cascades after a concussive event: fever and edema.

Based on official data (US Department of Defense [DOD] 2016), more than 80% of the 14,549 reported cases of TBI in the Army last year were concussive events. TBI can be thought of as a 2-phase process: The initial trauma can cause damage to blood vessels, axonal shearing, degradation of the blood–brain barrier (BBB), neuronal cell death, and other injuries, while the secondary-injury process is damage caused by the body's response to the initial trauma and occurs in the hours and days following the traumatic event. The degradation of the BBB (Readnower et al. 2010) and damage to neuronal axons (Smith et al. 2003) contribute to TBI-related changes in the brain's transcriptome, including increased expression of pro-inflammatory cytokines and chemokines (Redell et al. 2013). These effects are typical responses of the body's activation of the immune system that can lead to swelling and cell death that contribute to a subset of the secondary injuries.

Microglia play a central role in mediating the secondary injury cascades. As the brain's resident macrophage, microglia typically exist in a "sensing" phenotype that has a characteristic ramified appearance and features active, motile processes. When membrane receptors are activated, second messenger cascades transform microglia into an "activated" phenotype with an amoeboid appearance. Activated microglia migrate to the site of injury (damaged BBB, sheared axon, etc.) and perform functions common to the peripheral immune system. This project aims to

block some elements of activated microglia to prevent the production and release of pro-inflammatory cytokines and chemokines without reducing the activation, migration, or release of chemical signals that support healing and recovery.

To assess the effectiveness of novel biotechnology at minimizing inflammation in the brain, small changes in chemokines and cytokines must be quantified from microliters of biological samples (cerebral spinal fluid [CSF], brain tissue, blood plasma, etc.) in an animal model of TBI. Specifically, the larger Director's Research Initiative project (Whitaker and Harvey 2015) requires precise quantification of a range of different chemokines and cytokines related to inflammatory and pro-growth processes to within 10 pg/mL from sample volumes of 2–25 μL. There is some flexibility in which chemokines and cytokines are specifically used as endpoints, though they must be relevant for activation of microglia. Due to limited manpower, any automated system capable of meeting these requirements needs to be user friendly and contain safeguards against human error.

The Luminex microspheres are ideally suited for quantification of multiple proteins from a single, small volume sample. Briefly, superparamagnetic microspheres or "beads" are microparticles that include 2 specific fluorophores that give them identifiable optical properties. These beads can be coated with antibodies that specifically and irreversibly conjugate to a protein of interest within a complex matrix. This conjugate can be labeled with secondary, biotinylated antibodies that conjugate to streptavidin-phycoerythrin (PE) similar to sandwich enzyme-linked immunosorbent assays (ELISAs). The 3 fluorescent markers (2 beads plus PE) allow for extremely high resolution quantification of proteins using optical measurements. Through different combinations of the 2 fluorophores, beads of different "flavors" that are protein specific can be mixed within a single sample. Luminex, however, does not produce beads that are conjugated with antibodies but instead relies on vendor partners to provide them. The number of beads that can be used in the same sample volume is only limited by the bead reader.

Luminex has 3 different platforms for quantifying its beads: MagPix, 100/200, and FlexMap. The MagPix is the simplest, smallest, and least expensive platform. It uses a magnetic plate to create a monolayer of beads that can be imaged with a light-emitting-diode-based imager capable of differentiating up to 50 flavors of beads. The Luminex 100/200 creates a stream of beads that are read, separated, and quantified as a stream using laser-based imagers (similar to flow cytometry). Based on a similar mechanism, the FlexMap 3D is the most advanced system and is able to differentiate up to 500 bead types with greater speed for high-throughput applications. For this project, 2 vendors met the basic requirements of producing beads conjugated to antibodies that recognize rat inflammatory proteins: R&D

Systems and EMD Millipore's Milliplex system. The purpose of this project was to evaluate and compare the Luminex-based systems from R&D Systems and Milliplex for measuring chemokines and cytokines in biological samples from relevant, on-going research projects.

2. Methods

All of the samples used for these experiments were collected under protocols approved by the Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA). To determine the feasibility of using different multiplexed assays to quantify changes in proteins related to microglia activation after a TBI, samples from rat tissue and cell cultures were generously donated from other ongoing research projects to subjectively evaluate the relative workflows of the R&D Systems and Milliplex Luminex-based multiplexed protein assays, as well as semiquantitatively measure differences between samples.

2.1 R&D Systems

R&D Systems produces a single kit that works for rats. Within this kit, up to 17 protein analytes can be selected for quantification within each sample: CXCL2, CXCL3, GM-CSF, ICAM-1, IFN-gamma, IL-1 alpha, IL-1beta, IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, L-Selectin, TIMP-1, TNF-alpha, and VEGF. These analytes are generally associated with activation and aggregation of macrophage and microglia that lead to inflammatory and pro-growth immune responses. The kit also contains buffer solution, beads conjugated to antibodies, wash buffers, a positive control sample, and a standard agent with diluent to create the standard curve necessary to convert between optical measurements and protein concentrations.

To evaluate R&D Systems' Magnetic Luminex Screening Assay Rat Premixed Multi-Analyte Kit, a kit was purchased that included all of the 17 analytes included in company's catalog at the time. Samples were run in duplicate on the "black 96" well plate provided with the kit, along with the negative control (processed as normal, but with no sample added to the wells) and 6 dilutions of the provided standard complex. The experimental samples consisted of cell culture supernatants from CHME-5 (Janabi et al. 1995) and hu-YGM lines (generously provided by David Alvarez and Jon Karn of Case Western University) and rat and human microglia cell lines, respectively. These cells were chosen as candidates for future RNAi screening experiments. In addition to the parental CHME-5 cells, 4 sublines (denoted E3, E9, D10, and F3) were created from transfections of the parental CHME-5 line using a modified human immunodeficiency virus (HIV) provirus and were run in parallel with the parental line. For each cell line, culture supernatants

were collected at 3 points: from resting cultures, after 4 hours of lipopolysaccharide (LPS) treatment, and after 24 hours of LPS treatment. LPS was purchased from Sigma-Aldrich (St. Louis, Missouri) and used at 100 ng/mL.

The Luminex assay was conducted according to the vendor's protocol. Briefly, beads were reconstituted and added to each well of the microplate. Samples were added and mixed for 2 h at room temperature. Beads were held in place by a handheld magnet and washed with the provided buffer. Biotinylated antibodies were presented to each well and incubated for an hour. After another wash, the streptavidin-PE was added and incubated with the mixture according to manufacturer's instructions. After a final wash, the plate was read with the settings provided by the manufacturer with each kit.

There were technical difficulties with the backup demonstration unit on loan from Luminex. The plates were ultimately read on a Luminex 100/200 machine generously lent to us by colleagues in another institute.

2.2 EMD Millipore's Milliplex

The Milliplex system from EMD Millipore (now MilliporeSigma, the American life-science business of Merck KGaA) has many more options for analytes spread across several separate kits. For rats, there are 7 separate kits that feature 6–46 analytes to select from. The largest selection is within the cell-signaling kit, which is tailored to intracellular proteins and requires an overnight incubation step that the other kits do not. This kit does not include reagents to generate a standard curve and cannot quantify protein concentrations, which makes the results relative and limits utility for the larger project. The separation of analytes across different kits means that some samples need to be run multiple times to assay all analytes. For example, arginase 1 (ARG-1) is a marker for microglia in the brain and also a marker for the production of urea in the body. It is included in the Liver Injury Kit but not the Cytokine/Chemokine Panel. Twice as much sampling and effort is required to quantify ARG-1 and inflammatory markers with separate kits.

We used Milliplex's extensive library of analytes that can be measured in rat samples to explore the potential to quantify markers of microglia activation: ARG-1 and nuclear factor KAPPA-light-chain-enhancer of activated B cells (NFκB). ARG-1's function in activated microglia is not well established, and it is included on a Milliplex panel designed to test liver function. In the liver, ARG-1 is the enzyme that governs the final step in the production of urea; hence, it is included in the Rat Liver Injury Kit (No. RLI1MAG-92K). NFκB is a general transcription factor, and in microglia it functions to influence the regulation of gene transcription related to inflammatory processes. It is included in the Rat Cell Signaling Buffer

and Detection Kit (No. 48-602MAG). In addition, 6 of the 27 analytes available in the Rat Cytokine–Chemokine Microbead Panel (No. REECYTMAG-65K) were tested: IL-1α, IL-1β, IL-6, IL-10, IFN-γ, and TNF-α. This restriction to a subset of markers was required to manage costs.

CHME-5 cells were analyzed with the Milliplex kits to compare to the samples run on the R&D Systems kit. However, to optimize the utility of the time and resources, a wide range of sample types was also run. The same samples (immortalized microglia cell cultures, brain tissue homogenate, liver tissue homogenate, blood plasma, CSF) were used between all 3 Milliplex kits, but subsets of each type of sample was used for each kit to optimize the expected return on investment. All 3 kits were used at the same time, with several technical personnel working side by side. This led to several sources of human error that lowered confidence in the final quantitative values observed. The plates were read on the MagPix reader after software updates and general hardware maintenance from Luminex technicians.

2.2.1 Milliplex Cell Signaling Buffer and Detection Kit: NFκB

NFkB is a transcription factor known to be strongly activated in microglia that are producing inflammatory proteins. The Cell Signaling Buffer and Detection Kit's protocol requires a much longer incubation of the beads with the samples—overnight—than any other kit. This kit included a negative and positive (HeLa cells) control sample, but no standard curve. Without a standard curve, it was impossible to convert the optical measurements into concentration estimates. Since only one analyte was being used within the kit, this worked out to a set of expensive, problematic, and subjective ELISA. The space on the black-96 well plate was split between cell-culture samples, CSF samples, and blood-plasma samples with a few wells set aside for tissue homogenates. Based on previous results with the CHME-5 cells, immortalized murine microglia (BV-2) cell cultures were included on this plate instead of hu-YGM cell cultures. While reading this plate on the MagPix, the probe poked cleanly through the Mylar bottom of each well, destroying the samples. Following this incident, the probe's height was properly adjusted for the other 2 assay plates.

2.2.2 Milliplex Rat Liver Injury Panel: ARG-1

The workflow for the Rat Liver Injury Magnetic Bead Panel is similar to the kit from R&D Systems. In addition to the negative control, the standard curve included a seventh dilution. There were also 2 quality-control samples. When performed in duplicate, there was room on the plate for 38 experimental samples. To determine how this kit worked with a variety of sample types, several different kinds of samples were used with the same kit. The majority of the samples were rat plasma

taken from animals given a specialized diet for 10 or 17 days. The next-most frequent sample was liver homogenate. The last row was used to test one sample each of CHME-5 cell supernatants, BV-2 cell cultures, rat CSF, and rat-brain homogenate.

2.2.3 Milliplex Rat Cytokine/Chemokine Panel: IL-1 α, IL-1β, IL-6, IL-10, IFN-γ, and TNF-α

Similar to the Rat Liver Injury Panel, the Rat Cytokine–Chemokine Magnetic Bead Panel contained a negative control, a 7-sample standard dilution, and 2 quality-control samples. Since this kit was multiplexed, the standard master mix is actually a mixture of 3 different standards that must be checked to calculate each respective analyte (similar to R&D Systems' standard). For this kit, one sample of liver and one sample of brain homogenate were used. For cell cultures, cell lysates from CHME and BV-2 cells were used (control and LPS-treated). Many of the plasma samples from the Liver Injury Panel were run with this kit, as well. The majority of samples were CSF (1.5 uL each) from time-series experiments.

3. Results

All of the following values are means of the fluorescence measured from each sample, minus the median background fluorescence, plus or minus the standard error—unless otherwise noted.

3.1 R&D Systems Kit

For the majority of samples and analytes, it was not possible to reliably quantify the amount of protein present in the sample as the optical measurements taken were outside the range of the standard curves (Table 1). This is not surprising considering no optimization was conducted before running this kit and that the protein concentrations of the starting samples were not known. The results of the raw fluorescence measurements indicate the microbead assay performed as expected with high sensitivity. The median fluorescence of the duplicate measurements for each sample is reported here.

Table 1 Normalized median fluorescence data for 17 analytes in different cell cultures treated with LPS for 0, 4, and 24 h

		Parental CHME-5			E3			E9			F3			D10			hu-YGM		
		Rest	4 hr LPS	24 hr LPS	Rest	4 hr LPS	24 hr LPS	Rest	4 hr LPS	24 hr LPS	Rest	4 hr LPS	24 hr LPS	Rest	4 hr LPS	24 hr LPS	Rest	4 hr LPS	24 hr LPS
Pro-inflammatory	TNF-alpha	-13.7	-13.5	-13	-13.5	-13.7	-13.2	-15	-13.5	-14	-13.5	-13.7	-14.5	-12.7	-13	-13.5	-13.5	-12.5	-13.5
	IL-1a	-20.7	-20	-20.7	-20	-20.5	-20.7	-19.5	-20.7	-20	-19.5	-19	-18.5	-20.5	-20.5	-20	-21.5	-19	-20
	IL-1B	-64.8	-62.5	-61.8	-65.1	-61.5	-66.6	-63.8	-60.5	-62.3	-60.5	-59.3	-62.5	-62.3	-63	-62	-62.3	-56.3	-61
	IL-6	-33.2	-32.7	-31	-33	-32	-35.5	-32	-31.2	-34.5	-28	-32.2	-33	-33.5	-32.2	-33.5	-31.5	-31.5	-32.7
	IFN-G	-170.5	-164.8	-155.8	-175	-176.5	-189.5	-177.5	-174.8	-188	-161.5	-169	-184.3	-167	-177.5	-178	-172.3	-176.8	-187.8
	IL-18	-17	-22.3	-21	-23.5	-25.6	-25.8	-20.5	-24	-24.3	-24.8	-25.3	-26.6	-7.3	-23.3	-22.3	-24.8	-25.6	-25.6
	ICAM-1	66	19	83.7	96.2	18	91	89	17.5	97	95.7	12.5	92	53	5	46.7	-10.1	-9	-9.6
	CXCL-2	-24.5	14.5	134	-23.5	122.5	214	-24.5	22	149.2	-24.8	18	76.2	-25.8	-25.6	-24.3	-25.6	-24	-26.1
Non-inflammatory	IL-4	-16.5	-19	-17.5	-18	-18.5	-19	-17.5	-17.5	-17.5	-17	-18	-18.5	-18	-18	-18.2	-19.3	-18	-19
	IL-13	-28.8	-26.5	-27.7	-29.8	-29.5	-29.5	-30.5	-27.5	-30	-28.5	-26.7	-29	-30.5	-27.5	-30	-28	-26.7	-29
	IL-2	-27.5	-24.5	-26	-27.5	-25.5	-28	-28	-23.5	-27.5	-26	-22.8	-26	-26.5	-26	-27	-24	-22.3	-21
	IL-10	-40.5	-35.5	-38.5	-38.3	-40	-40.8	-40	-37.5	-40.3	-36.8	-33.3	-37.8	-40.5	-38	-39.5	-36.3	-33.3	-32.7
	VEGF	5838	3366.5	5870	5182.8	2050.5	4392.8	6098.5	3180.5	5695.5	5218.3	2141.3	4270.5	5295.8	1954.3	4973	452.5	63.5	228.3
Cell Adhesion and Aggregation	L-Selectin	-17.5	-17	-16	-16.5	-18	-18	-17.5	-17	-17	-16.7	-18	-17.5	-17.3	-17	-17.5	-18	-16.5	-18
	GM-CSF	-17.7	-18	-18	-18	-18.5	-18.5	-18.5	-18.7	-17.2	-16	-18.5	-19	-18	-18.5	-19	-20	-18.5	-19
	TIMP-1	34	1.3	44.5	25.8	-3.7	16	92.5	4.8	102.3	-6	-11.5	-5.5	9314	1588	8793.8	-13.5	-12.5	-12.5
	CXCL-3	-12.2	1012.5	2044.8	-12	1587.3	2225.3	-9.5	1498	3784.8	-13	130.8	537.8	-8.2	-8	3.3	-14	-12	-13.5

Note: Values are the median of technical duplicates, with the median of the background measurements subtracted out.

Three analytes yielded relatively high fluorescence across the cell lines: VEGF, CXCL-3, and TIMP-1 (as seen in Fig. 1) though the TIMP-1 was only high in the CHME-5 (D10 line).

In Fig. 1, analytes have been grouped according to a rough classification of function: inflammatory, noninflammatory, or "other"). This is a gross classification based on the description in the National Center for Biotechnology Information's database for each protein. As shown in Fig. 1, the R&D Systems multiplexed protein assay for rodents can be divided into pro-inflammatory, noninflammatory, and "other" types of proteins. The other proteins are related to cell adhesion and aggregation in the immune response, but are not directly linked to inflammatory processes. Data are presented as median value of duplicate samples, normalized by subtracting the median of the 2 background samples.

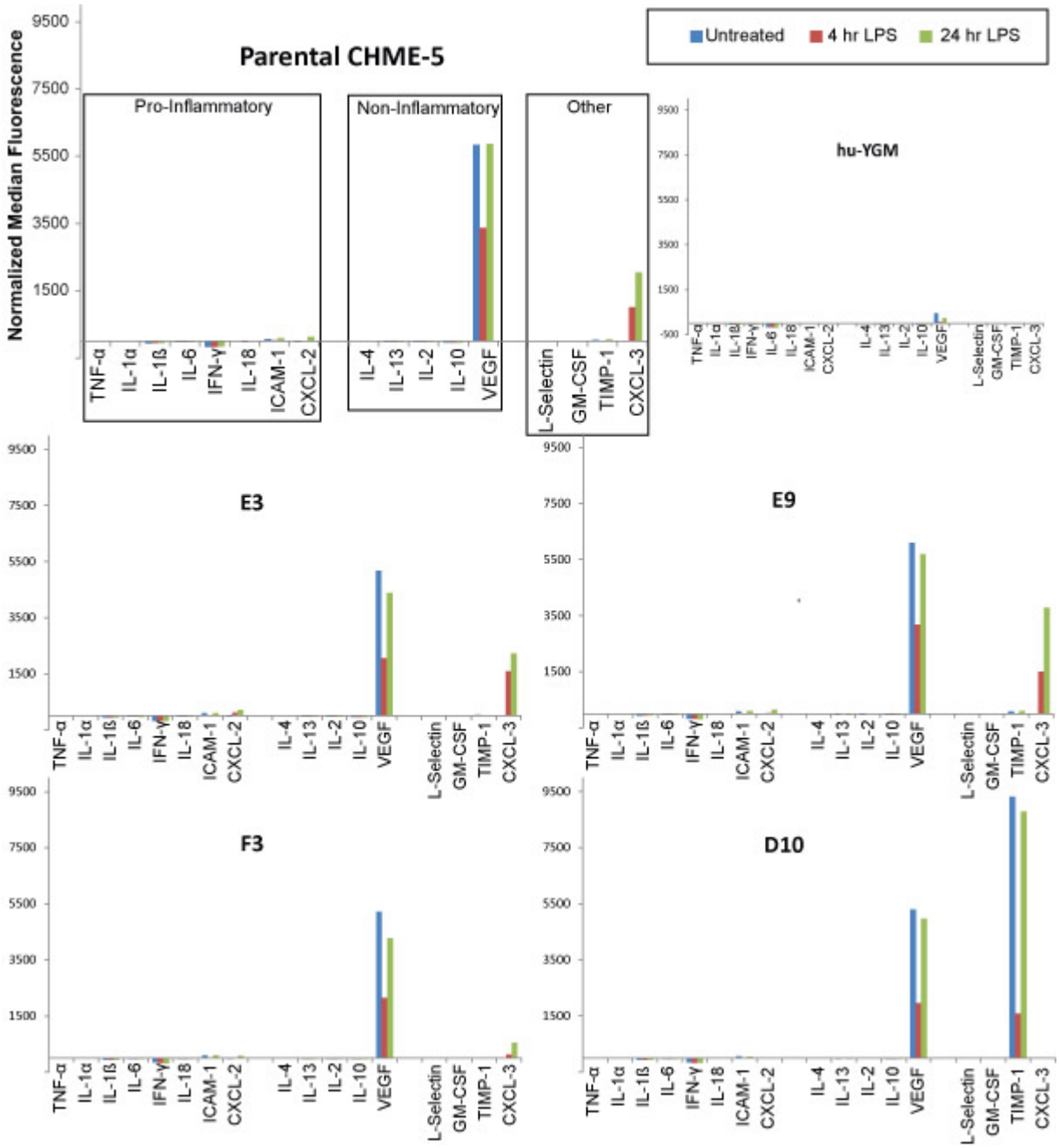

Fig. 1 Analyte results using the R&D Systems Kit on CHME-5 microglia: Parental CHME-5 cell line is an immortalized mouse microglia culture transfected to include components of HIV to generate sublines E3, E9, F3, and D10. Hu-YGM is a human-derived microglia cell culture included as a negative control. Since this assay is optimized for rat, the antibodies were not expected to bind to the human variants. The D10 subline produced a high signal to TIMP-1, but not CXCL-3, when the other lines were observed to have the opposite profile. VEGF also produces a strong fluorescent signal.

Figure 2 shows the CHME-5 inflammatory profile using the R&D Systems Kit. Focusing on the pro-inflammatory markers, it is notable that ICAM-1 and CXCL-2 are above background levels. CXCL-2 shows the expected increasing concentration with longer exposure to LPS. For the hu-YGM cell line, all analytes were below the level of background. Figure 2's data are presented as median value of duplicate samples, normalized by subtracting the median of the 2 background samples.

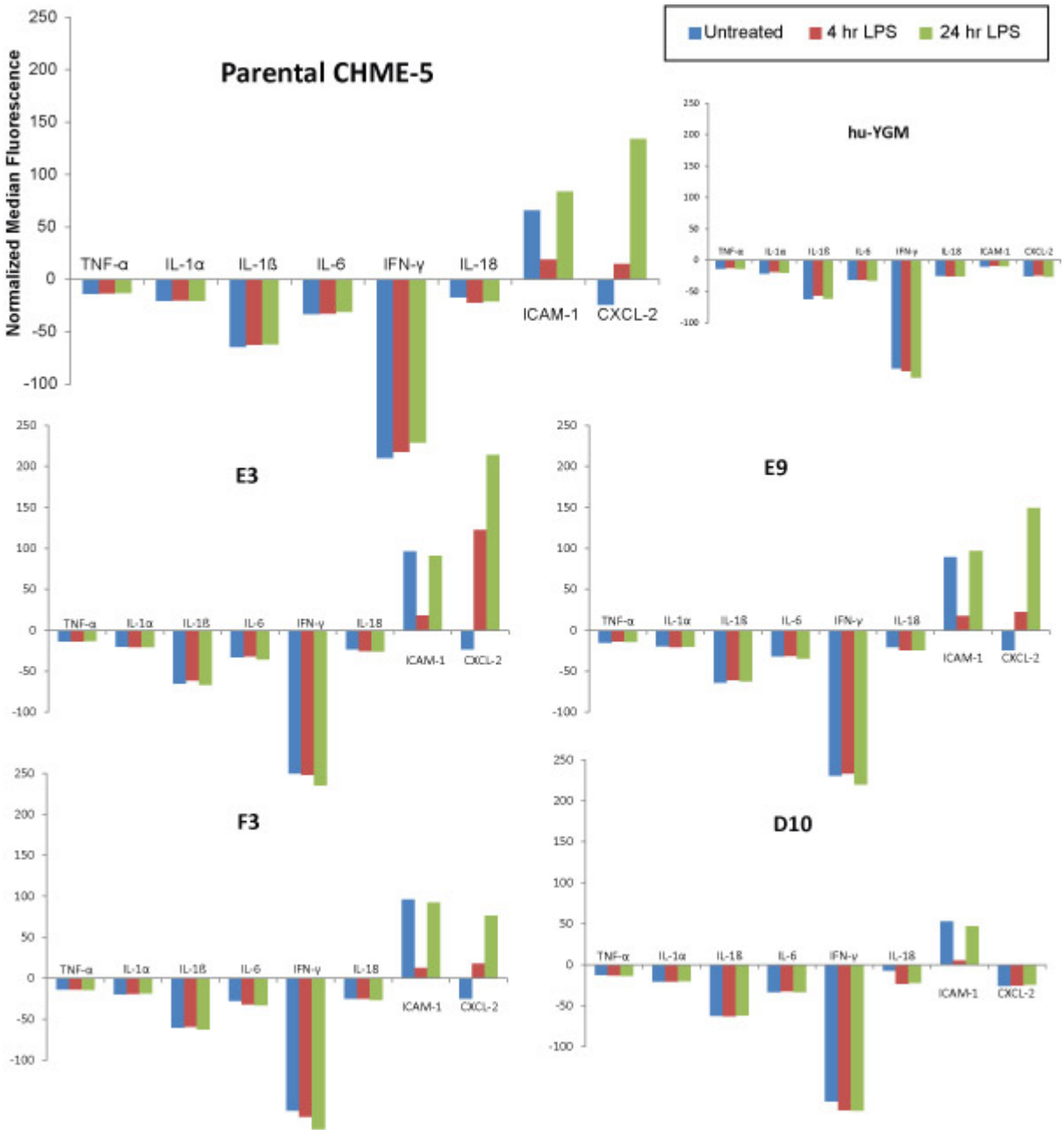

Fig. 2 Focusing on Fig. 1's pro-inflammatory subset of proteins, the majority of the fluorescence measured was below the level of background signal. ICAM-1 and CXCL-2 did show some signal above background levels after LPS treatment.

3.2 Milliplex Kits

For the Milliplex kits, a variety of tissue types were run to evaluate how well the kits tolerated matrix effects. Arguably, the most important thing to note is that the standard solution provided with 2 of the kits (Rat Liver Injury Panel and Rat Cytokine–Chemokine) to generate the standard curve that allows for quantification from optical measurements should be diluted in the matrix that matches the samples (i.e., serum matrix for serum, homogenization buffer for tissue homogenates, assay buffer for CSF, media for cell culture). For this set of experiments, standards were diluted in assay buffer as described in the protocol from the vendor. Therefore, the results discussed here are restricted to the optical-measurement data (as seen in Table 2). However, it should be noted that the results from the quality-control samples did not fall into the ranges provided with each kit.

Table 2 Normalized median fluorescence data for 8 analytes across 3 separate kits

			IL-1a	IL-1b	IL-6	IL-10	IFNg	TNFa	NFkB	ARG1
Microglia Cell Culture	Parental	Rest	-2.5	-1	-1	-2	-7	-0.5	90.25	
	CHME-5	LPS treated	-5.25	-3	-4	3	-24	-1.5	127.75	50.5
	E3	Rest	-3.5	0	-1	-1	-15.5	0.5	63	
	CHME-5	LPS treated	-1.5	0.5	-1	10.5	1.25	-1	53.5	
	E9	Rest	-2	0.5	0	1.25	-5.5	0	96.25	
	CHME-5	LPS treated	2.5	4	2	14.5	6	0.5	81.25	
	F3	Rest	-4	-1.5	-1.5	-3.5	-14.5	-1	122	
	CHME-5	LPS treated	0.25	2.5	1.5	10.5	1.75	1	115.75	
	D10	Rest	-6.5	-4	-3.5	-7	-29.5	-2	130.5	
	CHME-5	LPS treated	3	4	2	9	10.5	1	123.5	
	BV2	Rest	-3	-0.5	-2	-0.5	5	9.5	266	
		LPS treated	12	8.25	6.5	101.75	82.5	5525.75	91.5	65.5
Plasma	235	10 days	-2	4	-7.5	-1.5	-8.5	0		1
		17 days	-7	11	-7.5	-1.5	-17	0.5		0
	236	10 days								3
		17 days								18
	237	10 days								1.5
		17 days								-0.5
	238	10 days								1.5
		17 days								0
	239	10 days	-9.5	11	-7	-1	-1	-0.5		5.5
		17 days	-12	3.5	-8	-3	-20	0		8.5
	240	10 days								3.5
		17 days								0
	241	10 days								-0.5
		17 days								1.75
	242	10 days								3
		17 days								1.5
	243	10 days								-0.5
		17 days								8.5
	244	10 days								8.5
		17 days								2.75
	245	10 days								-0.5
		17 days								0.5
	246	10 days								1
		17 days								8.5
	247	10 days	-7.5	14.5	-6.5	3	-2	1		-0.5
		17 days	-10	6.5	-7.5	0	-30	0.5		0
	248	10 days								0.5
		17 days								5.5
Liver Homogenate	236		101.25	332.5	17.5	308	200.5	12		4656
	237									4311.5
	239									4441.75
	244									4129.5
	246									4109.5
Brain Homogenate	1		32	110.5	18.5	102.5	258	5.5	52.5	
	2								45.5	
	3								74	
Cerebral Spinal Fluid	22649	Background 1	6	5.5	3	9	40.5	0	4	
		Background 2	5	4	4	8.5	34.5	0	8.5	
		Background 3	10.5	8.5	6	16.5	68.75	0.5	13	
		Methamphetamine	11	7.25	6.5	14	79	0.5	2	
		Recovery	-0.5	1	-6	-2	-45	-3.5	5.5	
	22652	Background 1	8.5	8.5	4.5	14.5	55.5	-1	3	
		Background 2	4	2.5	2.5	5	27	-1	2	
		Background 3	10.5	8.5	5	14	71	0.5	8.5	
		Methamphetamine	11	9	5.5	14.5	76.5	0.5	12.5	
		Recovery	4	6.5	1.5	8.5	33.5	0	2.25	
	22654	Background 1	5	4	3.5	9.5	23.5	-0.5	3.5	
		Background 2	0.5	1	1.5	2.5	3	-0.5	3.5	
		Background 3	8.5	4.5	5.5	11.5	46.25	0.5	10	
		Methamphetamine	9.5	5	5	14.5	56.5	0	14	
		Recovery								
	22659	Background 1	14	11.5	7.5	20.5	98.5	1.5	4.5	
		Background 2	12	10	7	16.75	82.5	1.5	5.25	
		Background 3	9	8.5	3.5	13.75	64.5	0.5	14	
		Methamphetamine	3	7	3	15.5	55.5	0	21	
		Recovery								

3.2.1 NFκB

Above-background levels of fluorescence were observed in 25 uL of rat-liver tissue homogenates (124.1 ± 82.8), rat-brain tissue homogenates (57.3 ± 18.8), CHME-5 and BV-2 cell culture samples (113.4 ± 80.4), and rat CSF (7.6 ± 6.1). However, the majority of the CSF samples had fluorescence values close to the negative control (background) samples. The quantification was precise enough to note differences over time in the liver samples, though human error eliminated the possibility of determining which of the 2 samples from each individual were collected at the 10- or 17-day time points. Therefore, they are reported here as "high" and "low" and the data are not included in the table. The cell-culture samples also yielded strong fluorescence relative to the background samples (113.4 ± 80.4), with only 10 uL of sample. CSF samples were much lower in fluorescence (7.6 ± 6.1), due in part because only 2 uL of sample was used. When adjusted by the dilution factor of 12.5, the results are similar to other samples in Fig. 3, which depicts NF-κB activity using a Milliplex Kit across a range of tissue types. Data in Fig. 3 are presented as median value of duplicate samples, normalized by subtracting the median of the 2 background samples.

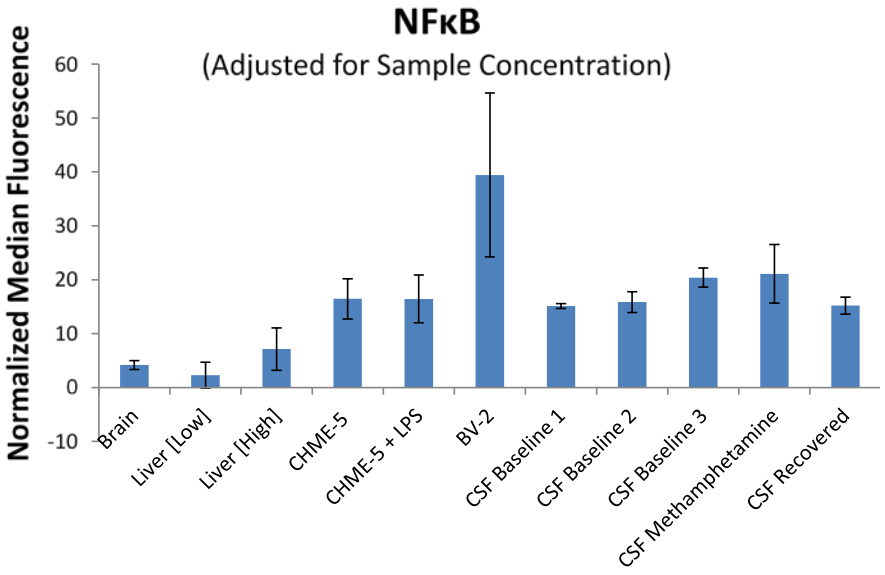

Fig. 3 Since different amounts of raw sample were used, the data shown have been multiplied by the inverse ratio of the amount of sample used. For example, since 2uL of CSF was diluted up to 25 uL of initial sample volume, the observed normalized, median fluorescence of 1.25 was multiplied by 12.5. This gives a rough estimation of the amount of fluorescence observed across the different sample types.

3.2.2 ARG-1

ARG-1 activity was measured using a Milliplex kit. The enzyme ARG-1 is a marker of microglia activation and the final enzyme in the production of urea. As shown in Fig. 4, intra-individual and interindividual differences are quantifiable from plasma samples, but the fluorescence values are much lower (2.9 ± 4.2) than observed in other types of samples (liver 4330 ± 228, cell culture 49 ± 17.3). The one CSF sample tested also resulted in a higher fluorescence measurement (31), even though only 2uL of sample was used compared to the 25 uL of sample used for all other samples. ARG-1 levels in CHME-5 and BV-2 cells treated with LPS were also detectable using this assay, with mean fluorescence values falling at 50 and 70 respectively.

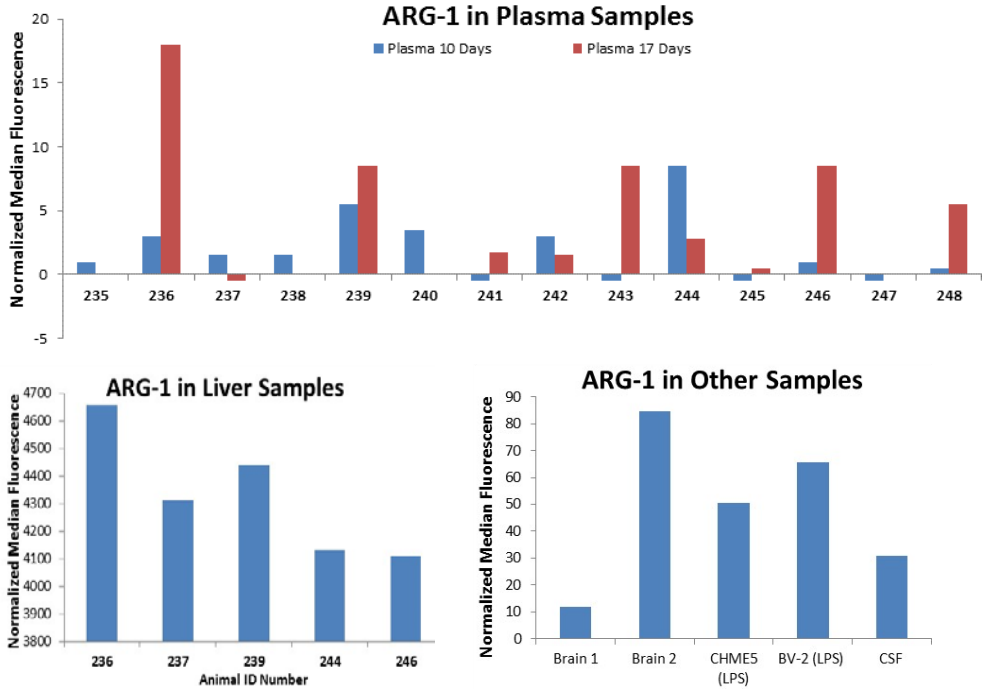

Fig. 4 Milliplex kit measured inter- and intra-subject differences in the amount of fluorescence from blood plasma, liver tissue, and "other" samples, which indicates an underlying difference in the amount of protein in each sample. Data are presented as median value of duplicate samples, normalized by subtracting the median of the 2 background samples.

3.2.3 Cytokines and Chemokines

The levels of IL-1α, IL-6, IFN-γ, TNF-α, and IL-10 in plasma were almost entirely below background levels. IL-1α and IFN-γ both decreased consistently across animals between Day 10 and Day 17, even though all observed fluorescence was below the level of the background. IL-1β was above background, but there was no consistent difference between samples from the 10- and 17-day time points (see Fig. 5's "Plasma Samples" depiction). These results are possibly the result of human error. Through a miscommunication, the initial sample concentration was below the recommended protein concentration required by the manufacturer.

Of the 2 cell-culture lines tested with this panel, the CHME-5 and BV-2 both had interesting trends. For the CHME-5 cells (see Fig. 5), even though the observed values are close to background levels, there is a consistent trend for LPS-treated subcultures to be above background and the untreated controls to be below the fluorescence measured for the background. It is unclear why this trend is not observed for the parental cell line. The subline specific nature of this result may be due to their stable transfection of a modified HIV provirus that may affect their LPS response. For the BV-2 cells, only the parental line has been tested. LPS treatment increased fluorescence for beads associated with every cytokine and chemokine included on this panel, particularly with TNF-α.

For the CSF samples that were tested, there is no obvious trend (as seen at the bottom of Fig. 5). IFN-γ has a generally high level of observed fluorescence, which is likely a technical artifact of the kit's reagents and not a biologically significant observation. The results do indicate there is a relative decrease in the second baseline measurement. The most noteworthy result is that quantifiable differences in fluorescence are observed at all because only 1.5 uL of each sample was used.

Figure 5's data—pro-inflammatory markers from use of the Milliplex kit—are presented as median value of duplicate samples, normalized by subtracting the median of the 2 background samples.

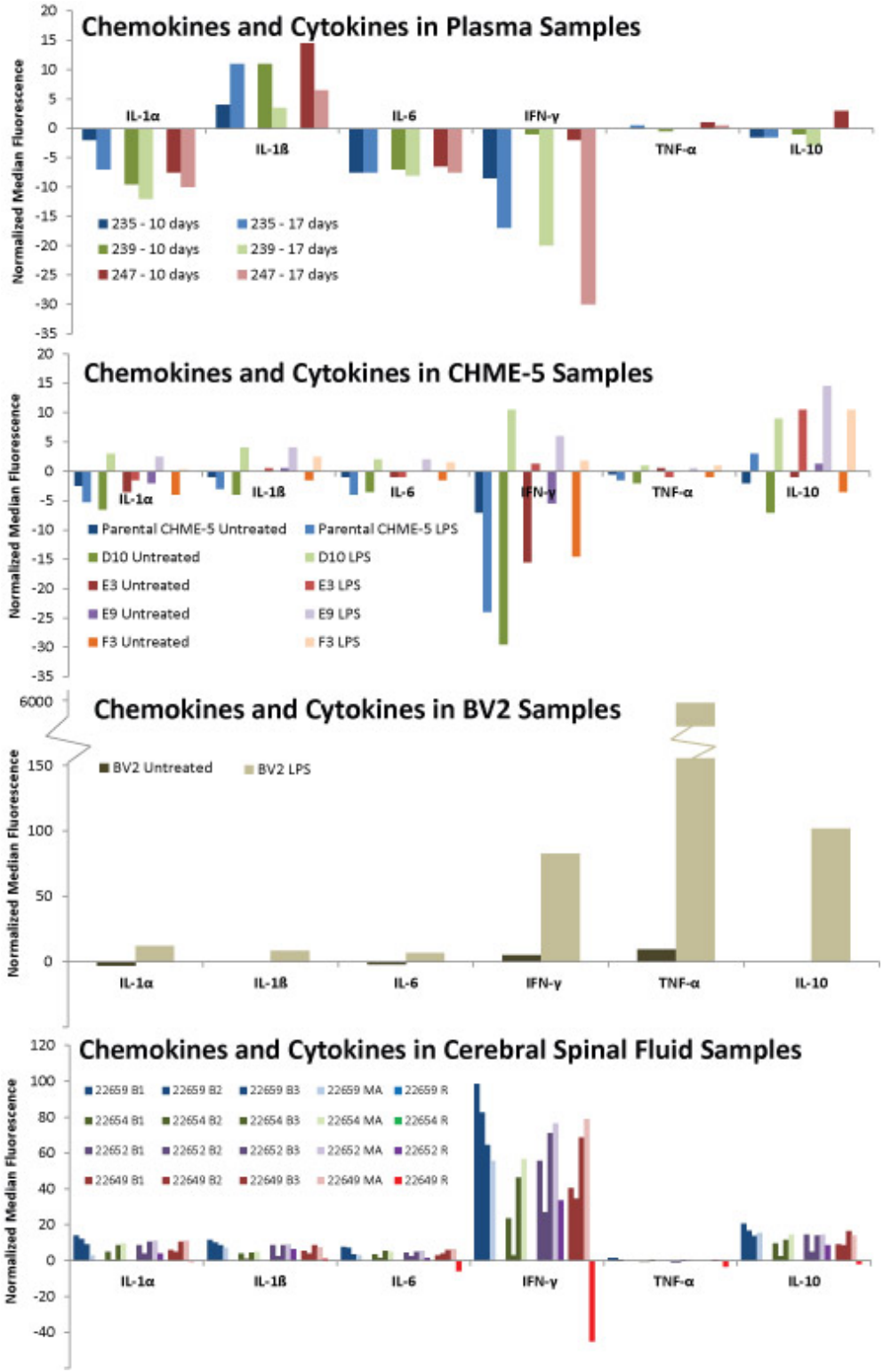

Fig. 5 A consistent, relative decrease in IL-1α and IFN-γ was observed among plasma samples taken from all 3 individuals at 10 and 17 days. Similarly, CHME-5 cells showed a relative increase in inflammatory markers after LPS treatment. This relationship as much more robust in BV-2 cells. No obvious trend was observed across CSF samples, though only 1.5 uL of sample was used for each measurement.

4. Discussion

Based on the results presented here, the decision was made to pursue acquisition of the R&D Systems products. The single kit resulted in fewer human errors than the multiple kits used for the Milliplex system and required a third of the sample volume. Although R&D Systems has far fewer analytes that can be assayed with the kits, it does have CXCL-3 (also known as "macrophage inflammatory protein-2-beta"), which may be important for studies involving microglia-induced inflammatory processes following TBI.

The Luminex system had several strengths and weaknesses that are worth noting for future experiments. First and foremost, the quantification of the amount of protein in the starting sample is a critical piece of information, which we learned after seeing the below-threshold results from the first kit run. Each kit, regardless of vendor, is optimized for a range of protein concentration expected in the sample. Although the manufacturer's protocol may include directions for dilution of the sample, these instructions should be altered to accommodate an initial concentration that is outside the expected range. For example, when running the blood-plasma samples (0.073–0.428 mg/mL), the samples were diluted 1:2 in buffer according to the manufacturer's instructions. When the samples were diluted again in the 96 well plate, the protein concentrations were too low for quantification by the reader. Proper dilution is not only crucial for assay readout but also for collection. The National Institutes of Health set survival guidelines for rodent bleeding based on circulating blood volume. Considerations should include animal size, sampling frequency, and optimal anticoagulant volume. The CSF samples (0.318–0.415 mg/mL), however, worked well at higher dilutions (2 in 25 uL).

Another important point for the Milliplex system is that the standard curves generated for each quantification kit are supposed to be created by diluting the initial standard with the appropriate matrix solution for the sample type being analyzed; that is, serum matrix for serum samples, homogenization buffer for homogenate samples, media for the cell-culture samples, and assay buffer for CSF samples. To quantify multiple-sample types on the same plates, as was done here, separate standard curves would need to be run for each sample type. This would dramatically decrease the number of wells available for actual sample analysis and is not practical. The kit from R&D System does not have this requirement. The standard is diluted in a provided Calibrator Diluent RD6-52. This is sufficient for serum, plasma, and cell-culture supernates. For the purposes of product evaluation, Luminex loaned us a used MagPix plate reader that had a few technical issues providing insight into overall care and maintenance. Initially, a software update was

required to run the baseline calibrations. Due to network-access restrictions, updating the software required a separate visit from a technician. Therefore, the kit from R&D Systems was read on a Luminex 100/200 system generously made available by colleagues in the National Institute on Aging. Once the MagPix was updated, it became clear there was also a hardware issue related to the alignment of internal components that required a second technician. When the system was fully operational, miscalibration of the height of the probe tip first resulted in no sample being picked up by the machine. Recalibration resulted in the probe piercing the bottom of the plate. Once set properly, the MagPix functioned normally though the quality control samples provided with the Milliplex kits yielded results that were out of the expected ranges.

Immortalized microglia cell cultures are a useful model system for early stage test and evaluation of biological manipulations. The experiments outlined here studied cell lines derived from 3 species: Human (hu-YGM), mouse (BV-2), and rat (CHME-5). Several distinct clones of CHME-5 were tested. These clones were derived from treating the parental line with a transfection agent for a separate set of experiments unrelated to ARL's efforts. Since the kits used for these experiments are designed for use in rodents, it was not expected the hu-YGM cells would lead to a positive signal and were considered to be a negative control. The results did show that BV-2 cells produce a robust inflammatory response in response to LPS that could be detected with the rat-optimized Milliplex kit (as seen in Fig. 5). The CHME-5 cells also resulted in a relative increase of cytokines and chemokines after LPS treatment, though the process needs to be optimized to increase the signal in future experiments.

The results on the presented cell lines are noteworthy due to the importance of microglia TBI. The inflammatory processes of activated microglia persist from months to years after the initial physical injury, thereby leading to a chronic degeneration or neurons in this area (Acosta et al 2013). Incidentally, it is important to note there is heterogeneity in microglia actions and effects during brain injury. Microglia that activate in the "M1-like" phenotype are typically related to pro-inflammatory processes. However, many microglia (deemed "M2-like") participate in protective actions including the resolution of inflammation, neurogenesis, and repair (Sica and Mantovani 2012). Therefore, enhancing the outcome of TBI may not simply result from decreasing inflammation, but enhancing the neuroprotective/neuroregenerative properties of microglia. By triggering RNAi to directly change the transcriptome of cells in the injury area, one may be able to differentiate recruited microglia to the M2 phenotype, thus enhancing repair. Overall, the characterization of several inflammatory markers in microglia through

the Luminex microspheres and described assays allowed us to utilize a high-throughput screen to identify resultant changes in microglia profile after shRNA addition.

5. References

Acosta SA, Tajiri N, Shinozuka K, Ishikawa H, Grimmig B, Diamond DM, Sanberg PR, Bickford PC, Kaneko Y, Borlongan CV. Long-term upregulation of inflammation and suppression of cell proliferation in the brain of adult rats exposed to traumatic brain injury using the controlled cortical impact model. PLoS One. 2013;8(1):1–8.

Blennow K, Hardy J, Zetterberg H. The neuropathology and neurobiology of traumatic brain injury. Neuron. 2012;76(5):886–899.

DoD Worldwide Numbers for TBI. Silver Spring (MD): Defense and Veterans Brain Injury Center (US); 2016 Jan [accessed 2016 Feb 10]. http://dvbic.dcoe.mil/dod-worldwide-numbers-tbi.

Hoge CW, McGurk D, Thomas JL, Cox AL, Engel CC, Castro CA. Mild traumatic brain injury in US Soldiers returning from Iraq. N Engl J Med. 2008;358(5):453–463.

Janabi N, Peudenier S, Heron B, Ng KH, Tardieu M. Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. Neurosci Lett. 1995;195(2):105–108.

Kelley AM, Athy JR, Cho TH, Erickson B, King M, Cruz P. Risk propensity and health risk behaviors in US Army soldiers with and without psychological disturbances across the deployment cycle. J Psych Res. 2012;46(5):582–589.

Logan BW, Goldman S, Zola M, Mackey A. Concussive brain injury in the military: September 2001 to the present. Behav Sci Law. 2013;31(6):803–813.

Readnower RD, Chavko M, Adeeb S, Conroy MD, Pauly JR, McCarron RM, Sullivan PG. Increase in blood–brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury. J Neurosci Res. 2010;88(16):3530–3539.

Redell JB, Moore AN, Grill RJ, Johnson D, Zhao J, Liu Y, Dash PK. Analysis of functional pathways altered after mild traumatic brain injury. J Neurotra. 2013;30:752–764.

Sica A, Mantovani A. Macrophage plasticity and polarization: in vitro veritas. J Clin Invest. 2012;122(3):787–795.

Smith DH, Meaney DF, Shull WH. Diffuse axonal injury in head trauma. J Head Trauma Rehab. 2003;18(4):307–316.

Vargas-Gonzalez LR, Gurganus JC. Hybridized composite architecture for mitigation of non-penetrating ballistic trauma. Intl J Impact Eng. 2015;86:295–306.

Whitaker KW, Harvey BK. 2015. Biological tools to optimize Soldier resiliency through epigenetic modification of microglia. Army Research Laboratory DRI16-HR-023. Aberdeen Proving Ground (MD).

List of Symbols, Abbreviations, and Acronyms

ARG-1	arginase 1
ARL	US Army Research Laboratory
BBB	blood–brain barrier
CSF	cerebral spinal fluid
DOD	US Department of Defense
ELISA	enzyme-linked immunosorbent assay
HIV	human immunodeficiency virus
LPS	lipopolysaccharide
NIDA	National Institute on Drug Abuse
PE	phycoerythrin
TBI	traumatic brain injury

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